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(54) Title: IMMUNOCYTOCHEMICAL STAINING OF EXFOLIATED CERVICAL SAMPLES

(57) Abstract

A method of identifying an individual with a squamous intraepithelial lesion is disclosed. The method comprises the step of immunocytochemically analyzing samples of exfoliated cervical cells from an individual. The sample is air dried or desiccated by a non-immunologically neutralizing fixative and contacted with detectable antibodies that bind to antigens that are expressed by proliferating epithelial cells. After incubating the antibodies with the sample and then removing unbound antibodies, the sample inspected to detect the presence of antibodies bound to antigen in epithelial cells which indicates a squamous intraepithelial lesion in the sample. The present invention provides a method of identifying an individual with a squamous intraepithelial lesion by performing immunocytochemical analysis in parallel with Pap smear analysis or following identification of individuals with atypical squamous cells of undetermined significance by Pap smear analysis.

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IMMUNOCYTOCHEMICAL STAINING OF EXFOLIATED CERVICAL SAMPLES

FIELD OF THE INVENTION

The invention relates to identification of pre-neoplastic and neoplastic squamous cell lesions of the human uterine cervix by evaluating immunocytochemically-stained samples of exfoliated cervical tissue, looking for abnormally proliferating squamous cells.

BACKGROUND OF THE INVENTION

Cytomorphologic interpretation of a Papanicolaou-stained cervical smear (Pap smear) is the mainstay of cytologic evaluation of the human cervix. The predictive value of the cytologic diagnosis of squamous intraepithelial lesion (SIL) is quite high (DiBonito L, *et al.* 1993 *Cancer* 72:3002-6). In SIL, cervical cytology serves as an excellent tool for identifying patients who need colposcopic evaluation and biopsy.

The cytologic diagnosis of squamous atypia, or "atypical squamous cells of undetermined significance" (ASCUS), is much less predictive. Patients with ASCUS may be managed by performing a repeat Pap smear - that may be subject to the same interpretive and sampling problems of the earlier smear (Busseniers A.E. and Sidawy M.K. 1991 *J. Reprod. Med.* 36:85-88 and Soutter W.P., *et al.* 1986 *Br. J. Obstet. Gynaecol.* 93:70-74) - or by colposcopy (Darnell Jones D.E., *et al.* 1987 *Am. J. Obstet. Gynecol.* 157:544-9 and Noumoff J.S. 1987 *Am. J. Obstet. Gynecol.* 156:628-31). From 17 to 68 percent of patients with

squamous atypia on cervical smears will harbor SIL on subsequent cervical biopsy performed at colposcopy. Viewed from the opposite perspective, 32 to 83 percent of patients undergoing cervical biopsy due to a cytologic diagnosis of ASCUS will harbor no pathologic changes. The aggregate associated costs for colposcopic evaluation and intervention to remedy these lesions approaches $6 billion annually.

Essentially, the current methods provide highly predictive means for screening individuals for cervical abnormalities. However, a cytologic diagnosis of ASCUS occurs in between 1.6 and 9.2 percent of all reported smears (Davey D.D., *et al.* 1994 *Mod. Pathol.* 7:43A). In patient populations with a high prevalence of ASCUS, the development of a reliable adjunctive test, possibly using immunocytochemical methods, might prove useful to identify patients with ASCUS who would benefit most from colposcopy.

There remains a need to develop more predictive methods for cytologic diagnosis of ASCUS. There remains a need to develop methods which can be used alone or in conjunction with Pap smear methodology to establish more definitive diagnosis when evaluating exfoliated cervical tissue samples.

SUMMARY OF THE INVENTION

The present invention relates to a method of identifying an individual with a squamous intraepithelial lesion. The method comprises the steps of first depositing a sample of exfoliated cervical cells from an individual onto a solid support and then fixing cells of the sample by non-immunologically neutralizing desiccation or a non-immunologically neutralizing fixative. The sample is then incubated with specific antibodies that bind to antigens that are expressed by proliferating epithelial cells for a time sufficient and under conditions suitable for the antibodies to form complexes with antigens present in the sample. Unbound antibodies are removed from the sample and the sample is inspected to detect the presence of antibodies bound to antigen in epithelial cells in the sample. The presence of antibodies

bound to antigen in squamous epithelial cells with enlarged nuclei in the sample indicates a proliferating cell population. The detection of this proliferating cell population indicates a high probability that a squamous intraepithelial lesion or squamous carcinoma is present.

The present invention relates to a method of identifying an individual with a squamous intraepithelial lesion. The method comprises the steps of: preparing corresponding parallel samples that contain exfoliated cervical cells. A first sample of exfoliated cervical cells from said individual is deposited onto a solid support and a second sample of exfoliated cervical cells from said individual is deposited onto a solid support. In some embodiments, the first sample of exfoliated cervical cells from said individual is deposited onto a first solid support and a second sample of exfoliated cervical cells from said individual is deposited onto a second solid support. In some embodiments, the first sample of exfoliated cervical cells from said individual is deposited onto a solid support and a second sample of exfoliated cervical cells from said individual is deposited onto an adjacent part of the solid support. The cells of the first sample are fixed with a Pap smear fixative that prepares them for Papanicolaou staining. The cells of the second sample are fixed by non-immunologically neutralizing desiccation or a non-immunologically neutralizing fixative. The cells of the first sample are contacted with Papanicolaou stain for sufficient time for the cells to incorporate stain and become stained. The excess stain is removed by washing to remove unincorporated stain and the sample is evaluated to detect staining patterns of cells consistent of Pap smear analysis to determine if the sample contains (1) normal cells only or (2) a squamous intraepithelial lesion or (3) atypical squamous cells of undetermined significance. If the Pap smear analysis results indicates atypical squamous cells of undetermined significance, the second sample is evaluated by the immunocytochemical assay. The immunocytochemical assay comprises the further steps of contacting the second sample

with detectable antibodies that bind to antigens that are expressed by proliferating epithelial cells for a time sufficient and under conditions suitable for said antibodies to form complexes with antigens present in the second sample. The unbound antibodies are removed and the sample is inspected to detect the presence of antibodies bound to the antigen in epithelial cells. The presence of antibodies bound to antigen in squamous cells with enlarged nuclei in the second sample indicates a proliferating cell population. The detection of this proliferating cell population indicates a high probability that a squamous intraepithelial lesion or squamous carcinoma exists.

DETAILED DESCRIPTION OF THE INVENTION

According to the invention, a method is provided which can identify proliferating cells in an exfoliated cervical tissue sample. When used in conjunction with other evaluation techniques, assays and analyses, the present invention provides methods of analyzing an exfoliated cervical tissue sample to obtain information with respect to the nature the cell proliferation, the molecular basis of the cell proliferation, the presence of malignant disease, the extent of development of disease. Using one or more assays described herein in combination with other assays, an exfoliated cervical tissue sample may be used to identify individuals with abnormally proliferating cells, to identify whether the proliferation is a benign hyperplasia or a malignancy, what the molecular nature and basis of the malignancy is and/or the extent of disease development. The invention provides the means to identify proliferation of cells in exfoliated cervical samples and to make qualitative assessments of them. The present invention provides methods of identifying SIL in an exfoliated cervical tissue sample.

According to the invention, a method of determining SIL in exfoliated cervical tissue samples is provided. The method may be used independent of Pap smear analysis, parallel

with Pap smear analysis on separate samples taken from the same patient or as a follow up analysis after ASCUS diagnosis.

Pap smear methodology is well known, widely used and readily available to those having ordinary skill in the art. Briefly, to take a Pap smear, exfoliated cells are removed from the exocervix and endocervix with a swab, brush, spatula, or other sampling device. The cells are wiped onto one or two glass slides, and thereafter immediately sprayed with one of a number of commercially available fixatives (such as Surgipath Cytology Fixative, Surgipath Medical Industries, Inc., Grayslake, IL) and transported to the laboratory. There, the slide is stained with one of a variety of commercially available Papanicolaou stains or modifications thereof. Slides are examined microscopically by a cytotechnologist to determine adequacy of the specimen, and to screen for the presence of any cellular abnormality. If the cytotechnologist does not find any cellular abnormality, the case is diagnosed as negative, a report is generated, and signed by the cytotechnologist. If the cytotechnologist detects a cellular abnormality, the slide is reviewed by a pathologist who interprets the cellular abnormality on the smear, a report is issued, and signed by the pathologist. The currently accepted reporting system for Pap smears (also known as cervical smears or Papanicolaou smears) is the 1991 Bethesda System (Kurman RJ, Solomon D. *The Bethesda System for Reporting Cervical/Vaginal Cytologic Diagnoses*. Springer-Verlag, New York, 1994). According to this system, squamous cell abnormalities on Pap smears should be classified into one of four diagnostic categories:

1) Atypical squamous cell of undetermined significance (ASCUS): cellular abnormalities qualitatively or quantitatively insufficient for a definitive diagnosis of a squamous intraepithelial lesion or squamous carcinoma.

2) Low grade squamous intraepithelial lesion (LGSIL): cellular abnormalities include nuclear enlargement (up to three times the area of normal intermediate nuclei), a mild to modest increase in nuclear/cytoplasmic ratio, moderate

variation in nuclear size and shape, rare or absent nucleoli, and nuclear hyperchromasia.

3) High grade squamous intraepithelial lesion: criteria of LGSIL but with a higher nuclear/cytoplasmic ratio, resulting in a cell size that is usually lower than seen in LGSIL. Nucleoli are generally absent and nuclear outlines are irregular. The cytoplasm may appear either lacy or keratinized.

4) Squamous cell carcinoma: frank malignant cytologic features (further explained in the reference by Kurman and Solomon).

The invention provides an immunocytochemical method of diagnosing SIL. According to the invention, antibodies which bind to antigens characteristically expressed by proliferating cells are contacted with samples of exfoliated cervical tissue for a time sufficient and under conditions in which the antibodies will bind to the antigens present in cells of the tissue sample. The antibodies that are used bind to antigens that are characteristically expressed by proliferating cells and that are not expressed by normal squamous intraepithelial cells. The antibodies are usually detectable by light microscopy. In particular, the antibodies are usually detectable by light microscopy due to a label conjugated directly to them or by contacting them with a second antibody that is specific for the first antibody and that is detectable such as by a conjugated label. Thus, the presence of antigens in cells in the sample may be determined by detecting antibodies bound to cells of the sample. The sample is examined to determine whether squamous intraepithelial cells in the sample contain the antigen.

According to the present invention, samples are obtained in the manner similar to that which is used to obtain Pap smear samples. That is, samples are collected using a swab, brush or spatula and cells in the sample are deposited on microscope plates by standard techniques well known to those skilled in the art. In some embodiments, a cytobrush is used to obtain endocervical samples that are smeared onto one half

of a slide, and Ayre spatulas are used to obtain cervical samples smeared onto the other half of the same slide.

Air-dried samples are obtained smearing collected exfoliated cells onto uncoated or NEOPRENE™ adhesive cement-coated glass slides. NEOPRENE™ adhesive cement (polychloroprene, Aldrich Chemical Company, Milwaukee, Wisconsin) may be used to promote cellular adhesion to glass slides. Other types of coated slides include poly-L-lysine coated slides, silanated slides and positively charged slides. Uncoated glass slides may be used for smears provided that the subsequent steps are not excessively disruptive to result in the detachment of the cells.

In some embodiments, samples may first be incorporated in a liquid medium for transport for example. Prior to analysis, the samples are transferred to a solid support.

In the case of a smear to be stained with the Papanicolaou stain, the sample is first fixed to the slide after it is deposited on a slide. The fixative used denatures proteins. Unlike those sample preparation methods followed in Pap smear collection, the samples used in the immunocytochemical assay of the present invention are not fixed in the manner used in Pap smear methodology. Rather, samples used in the immunocytochemical assay of the present invention are air dried and/or desiccated with a non-immunologically neutralizing fixative such as acetone. As used herein, the term "non-immunologically neutralizing fixative" is meant to refer to fixatives which do not render antigenic proteins non-antigenic by disruption of the conformation of recognized epitopes or otherwise. According to the invention, antibodies specific for a protein will recognize a protein after it has been exposed to a non-immunologically neutralizing fixative. Samples are air dried by exposing them to open air, a controlled gaseous environment and/or a vacuum or pressure deficient environment. Air dried samples are exposed to such environments until sufficient evaporation of moisture in the sample takes place. Samples prepared without immunologically

neutralizing fixative are referred to herein as "non-immunologically neutralized samples". Examples of non-immunologically neutralizing fixatives include acetone and alcohol such as ethanol.

As used herein, the term "antibody" is meant to refer to complete, intact antibodies, and Fab fragments and $F(ab)_2$ fragments thereof that binds to an antigen that is characteristically expressed by proliferating cells and that is not expressed by normal squamous intraepithelial cells.. Complete, intact antibodies include monoclonal antibodies such as murine monoclonal antibodies, chimeric antibodies and humanized antibodies. The production of antibodies and the protein structures of complete, intact antibodies, Fab fragments and $F(ab)_2$ fragments and the organization of the genetic sequences that encode such molecules are well known and are described, for example, in Harlow, E. and D. Lane (1988) *ANTIBODIES: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. which is incorporated herein by reference.

As used herein, the term "proliferating cell-specific antibodies" is meant to refer to antibodies that bind to antigens that are characteristically expressed by proliferating cells and that are not expressed by normal squamous intraepithelial cells.

The samples fixed with non-immunologically neutralizing fixative are contacted with proliferating cell specific antibodies. The conditions and amount of time sufficient for immunocomplex formation between the antibodies and antigens present in the non-immunologically neutralized samples can be determined using routine experimentation. Similarly, the techniques for detecting and visualizing any antibody-protein complexes of the immunocytochemically stained samples are well known. The conditions and times are dependent on the location of the antigen in the cell, i.e. cytoplasmic, cell membrane, nuclear membrane, nucleus. Incubation time is also a function of the affinity of the primary antibody for the

antigen, the concentration of the antibody, and the concentration of the antigen.

Examples of other antigens that are characteristically expressed by proliferating cells and that are not expressed by normal squamous intraepithelial cells include: Ki-67, cyclins, mutant p53 and other oncoproteins.

In preferred embodiments, assays are performed to identify other antigens after initial identification of the presence of Ki-67. Specifically, after identifying ASCUS cells by Ki-67 assay as being proliferating, the presence of other antigens can indicate the nature of the proliferation, the type of cancer which the patient may have and the extent of development of disease. Examples of other antigens that may be useful after triaging SILs by Ki-67 staining are cyclins, mutant p53 and other oncoproteins.

Ki-67 is a nuclear nonhistone antigen expressed in cells that are proliferating (Gerdes J., *et al.* 1984 *J. Immunol.* 133:1710-1715). The recent recognition of Ki-67 as an immunohistochemical marker of dysplasia in the cervix and vulva, where it is also present in normal basal and parabasal cells, makes it a potentially interesting marker to explore in cytologic material.

Proliferating cell specific antibodies may be commercially available. For example monoclonal antibody MIB-1, which binds to Ki-67, is commercially available from Immunotech Inc. (Westbrook, ME).

For the interpretation of Ki-67 immunostaining, any nuclear decoration in squamous epithelial cells were considered immunoreactive.

Proliferating cell specific antibodies must be detectable either by labelling them such as conjugating them to a detectable material, or by first contacting samples with proliferating cell specific antibodies and then, after removing any uncomplexed antibodies, contacting the sample with detectable antibodies that bind to the proliferating cell specific antibodies.

Importantly, labelling methods must be compatible with the samples to reduce or eliminate false labelling and ensure that labelled antibodies bound to antigen can be detected. For example, it has been discovered that prior to labelling with immunoperoxidase reagents (avidin-binding complex or streptavidin peroxidase), air dried smears must be pretreated with H_2O_2 in order to eliminate endogenous peroxidase in the sample. If the sample is not pretreated, the peroxidase in the sample activates the chromagen and the results are difficult to analyze (i.e. creating non-specific background staining).

Examples of labels for making antibodies detectable include avidin-biotin peroxidase (ABC), immunoalkaline phosphatase anti-alkaline phosphatase (APAAP), streptavidin peroxidase, streptavidin alkaline phosphate, glucose oxidase, fluorescein conjugates and immunogold to name a few.

As discussed above, the immunocytochemical assay is performed routinely. Proliferating cell specific antibodies are contacted with the smear a time sufficient and under conditions suitable for binding of the antibodies to any antigen present in the cells in the smear. After sufficient time has elapsed, the smear is washed to remove unbound antibody.

The smears are examined by a trained evaluator or an automated evaluator. Squamous cells in the smear are identified by their cytomorphologic characteristics. Examples of automated evaluators include the SAMBA 4000 (Imaging Products International Inc., Chantilly, IL) and Papnet (Neuromedical Systems, Inc. Suffern, New York). The squamous cells on the smear are evaluated for nuclear labelling by the chromogen detector as determined by the immunocytochemical methodology that was used. For example, diaminobenzidine\H_2O_2. (DAB) is commonly used as the chromogen for ABC. Following incubation with an antibody such as MIB-1 that labels proliferating cells, DAB decorates the abnormally enlarged squamous nuclei a dark brown color. The presence of abnormally

enlarged squamous nuclei that stain positively (e.g. are brown in color) would be considered a positive test.

The preferred embodiment of the invention provides the immunocytochemical assay as a secondary screening of samples following primary Pap smear evaluation. That is, two samples are taken from a patient: one is processed for Pap smear analysis while the other part of a processed for immunocytochemical analysis. The Pap smear sample is fixed with a denaturing fixative while the second smear is either not fixed (air dried) or fixed with a non-immunologically neutralizing fixative. The first smear and second smear are labelled so that they can be matched to each other as corresponding samples should parallel analysis be undertaken.

The first smear is fixed and stained as per standard Pap smear methodology and evaluated by a trained evaluator. Initially, the smear is evaluated by a cytotechnologist. If no cellular abnormality is identified, the smear is diagnosed as negative, a report is generated, and is signed by the cytopathologist. If a cellular abnormality is identified by the cytotechnologist, then the smear is evaluated by a pathologist. Currently, there is no FDA approved primary automated PAP smear evaluator. For those Pap smears that are interpreted as ASCUS by the pathologist, a second slide containing cervical cells may be evaluated by the immunocytochemical assay of the invention to diagnose SIL.

EXAMPLES

Example 1

Recent immunohistochemical studies performed in tissue sections have demonstrated that antibodies to proliferating cell nuclear antigen (PCNA) and Ki-67 decorate only the basal and parabasal nuclei of normal cervical and vulvar squamous mucosa. (Konishi I, *et al.* 1991 *Cancer* 68:1340-1350; Devictor B., *et al.* 1993 *Gynecol. Oncol.* 49:284-90; Karakitsos P., *et al.* 1994 *Gynecol. Oncol.* 55:101-107; Mittal K.R., *et al.* 1993 *Am. J. Surg. Pathol.* 17:117-122; Shurbaji M.S., *et al.* 1993 *Am. J. Clin. Pathol.* 100:22-26; Murakami T.,

et al. 1993 *Acta Obst Gynaec Jpn* 45:967-72; and Raju G. 1994 *Int. J. Gynecol. Pathol.* 13:337-41)

Ki-67 nuclear antigen is expressed in upper epithelial levels of intraepithelial neoplasia of the cervix and vulva, variably in condyloma, and in basal and parabasal cells of normal squamous mucosa in histologic preparations. The possible application of this pattern of immunoreactivity to cervical smears was explored using either 1) air-dried acetone-fixed cervical smears obtained from 106 consenting patients or 2) a single slide from archival 2-slide cases of SIL. MIB-1 monoclonal antibody to Ki-67 was tested using two immunocytochemical techniques.

In the development of this protocol, air-dried smears in addition to routine Papanicolaou-stained cervical smears were prepared only after obtaining consent from the patients. To our knowledge, this study was the first to attempt to apply immunocytochemical detection techniques to air-dried cervical smears. Although our laboratory and other laboratories have applied monoclonal antibodies to Ki-67 to air-dried aspiration smears and scrapes from non-gynecologic material (Henry M.J., *et al.* 1991 *Diagn. Cytopathol.* 7:591-596 and Liao S.Y. and E.J. Stanbridge 1995 *Mod. Pathol.* 8:42A), cervical smears presented unique difficulties that were not anticipated. In particular, H_2O_2/PBS blockade was found to be essential to facilitate a reliable interpretation of the slides. This step had not been performed in prior descriptions of Ki-67 immunostaining of cytologic material. In addition, the reliability of uncoated glass smears for cervical immunocytochemistry had not been tested.

Very few investigators have applied immunocytochemical stains to Pap smears. Immunocytochemical detection of MN tumor-associate antigen was studied in decolorized cervical smears. Although some antigens may be detectable using this method, in our experience with a small sample, detection of Ki-67 nuclear antigen using the MIB-1 antibody was not possible in decolorized smears. Thus, the collection of separate, air-dried smears was necessary to

achieve satisfactory results with the MIB-1 monoclonal antibody.

Additional cytologic specimens that contain diagnostic cellular material can be obtained from a single cervical spatula or brush without additional cervical scraping. In studies in which two samples obtained from the same scraping have been performed, there was no significant change in diagnostic sensitivity between the first and subsequent samples (Hutchinson M.L., *et al.* 1992 *Acta Cytol.* 36:499-504). It has been noted that at least 82 percent of the cells harvested following cervical scraping are thrown away with the discarded brush or spatula (Hutchinson M.L., *et al.* 1992 *Acta Cytol.* 36:577). Thus ample cellular material remains on the collecting device for additional smears to be made without additional cervical scraping or brushing.

MATERIALS AND METHODS

The study protocol was approved by the institutional review board of Thomas Jefferson University. All patients signed written consent for air-dried Pap smears to be taken and used for experimental purposes. Patients were enrolled for the study at the time of routine gynecologic examination, or at the time of colposcopic examination.

Air-dried Pap smears were obtained from 106 consenting patients and smeared onto Neoprene-coated glass slides. Neoprene (polychloroprene, Aldrich Chemical Company, Milwaukee, Wisconsin) is used in our laboratory to promote cellular adhesion to glass slides. In a limited number of cases, uncoated glass slides were used for smears. At least two smears were obtained from each patient.

A cytobrush was used to obtain endocervical samples that were smeared onto one half of a slid, and Ayre spatulas were used to obtain cervical samples smeared onto the other half of the same slide. Additional smears were prepared from each patient using the same spatula and brush sample from the first smear, without additional swabbing of the cervix.

Air-dried smears, labelled only with the patient's name, were desiccated, acetone-fixed for 10 minutes, and then

frozen at -70 degrees centigrade or immediately stained. Slides are stored in sealed slid boxes for up to three months before staining and the immunoreactivity is retained.

Archival Pap smears from 2-slide of each was obtained from file. The coverslip from one slide of each case was removed with xylene, and slides were destained using one percent acid alcohol, then rinsed in alcohol. Immunocytochemical stains were performed in this group of cases using the avidin-biotin peroxidase method with blockade of endogenous peroxidase.

The monoclonal antibody to Ki-67 antigen was MIB-1 which was obtained from Immunotech (Westbrook, ME). The antibody was used in a dilution of 1:50 to 1:100. Slides were incubated with the antibody for sixty minutes. Shandon Cadenza Immunostainers (Shandon, Pittsburgh, Pennsylvania) were used to perform the immunostaining, which was done at room temperature. This instrument has proven to be effective for immunocytochemical staining of cytospin preparations, peripheral blood smears, and touch preparations.

Two different immunocytochemical detection studies were tested on cervical smears. The first used an avidin-biotin peroxidase method (ABC) (Vector Laboratory, Burlingame, CA) for the detection of antigen-antibody complexes (Hsu S.M., *et al.* 1981 *J. Histochem. Cytochem.* 29:577-80) using 3,3'-diaminobenzidine (DAB)/H_2O_2 (Polysciences, Warrington, PA) as the chromogen. Peroxide (0.02%) and DAB (1 mg/ml) are combined 1:1. Briefly, this technique employs a biotinylated secondary antibody (such as for example horse anti-mouse) incubated for 30 minutes. ABC complex (peroxidase) is added for 30 minutes followed by the sequential application of DAB/peroxide substrate, three times for 8 minutes. Buffer washes (PBS pH 7.4 +/- 0.05) are applied between the primary antibody, secondary antibody, ABC and prior to the first DAB application.

The second immunocytochemical detection system used an immunoalkaline phosphatase anti-alkaline phosphatase (APAAP) method for detection of antigen-antibody complexes (Cordell

J.L., *et al.* 1984 *J. Histochem. Cytochem.* 32:219-229), employing new fuchsin as the chromogen.

In the subset of the cases in which the ABC technique was used, endogenous peroxidase activity was blocked by incubation in a 0.3 percent PBS/H_2O_2, pH 7.2 - 7.4, for 10 to 15 minutes. A Harris hematoxylin counterstain (Surgipath Inc.), blued with lithium carbonate, was applied. The slides were then dehydrated, cleared, and mounted.

For the interpretation of Ki-67 immunostaining, any nuclear decoration in squamous epithelial cells were considered immunoreactive. Abnormal squamous epithelial cells were those with enlarged nuclei, and often with irregular nuclear borders. Since air-drying artifact is commonly associated with artifactual nuclear swelling, the increased size of abnormal nuclei was assessed visually by comparison to neighboring normal superficial and intermediate cells.

RESULTS

Nuclear staining of abnormal squamous epithelial cells was identified in air-dried smears from patients with dysplasia using all of the above methods. ABC-stained slides that did not undergo blocking with H_2O_2/PBS were difficult to evaluate, because neutrophils and erythrocytes were reactive (brown), largely due to the presence of endogenous (pseudo) peroxidase. In areas of smears where neutrophils and/or erythrocytes were abundant and partially or largely obscured the epithelial component, these areas were extremely difficult to interpret. ABC-stained smears that underwent quenching of endogenous peroxidase were far easier to interpret. In these smears, immunoreactive squamous nuclei could be easily discerned from nonreactive squamous nuclei at 10X magnification. In smears where immunoreactive squamous nuclei appeared in fragments or were concentrated in one area, 4X scanning was sufficient to identify immunoreactive squamous nuclei, but higher magnification was necessary to verify their abnormal morphology. Even in thick areas of the smears, where conventional morphologic interpretation can be difficult,

bright brown, reactive nuclei could be easily distinguished from non-reactive, light blue nuclei.

In slides stained with the APAAP technique, abnormal squamous nuclei were immunoreactive, however, excessive staining in the cytoplasm of endocervical cells was noted. This excessive background reactivity rendered the smears difficult to interpret.

The morphologic identification of either ASCUS or SIL was possible in most smears and attributable, in large part, to nuclear enlargement. Koilocytes could generally be identified, and the nuclear/cytoplasmic ratio was easily discerned. Since nuclear chromatin detail was not always discernible in air-dried smears, chromatin texture was not evaluatable. Thus, despite the absence of optimal fixation and staining, the combination of morphologic features (i.e., nuclear size, nuclear/cytoplasmic ratio, perinuclear halos, nuclear membrane irregularities) and immunoreactivity enabled microscopic identification of abnormal squamous cells.

Smears made on Neoprene-coated glass slides and uncoated slides were comparable in cellularity and immunoreactivity. No immunoreactivity was identified in destained archival Pap smears.

DISCUSSION

The optimal protocol for identifying nuclear expression of Ki-67 using the monoclonal antibody MIB-1 utilized air-dried smears that were acetone-fixed, blocked with H_2O_2/PBS, and stained using ABC. Although most smears were collected on Neoprene-coated slides, uncoated glass slides performed equally well. The wash protocols of the Shandon Candenza immunostainer are not very rigorous, so coated slides did not make a difference. Manual techniques or other instruments may be more rigorous; there might be cellular loss on uncoated slides due to lengthy incubations in an aqueous environment.

The excessive cytoplasmic staining in the cytoplasm of endocervical cells might be attributable to endogenous alkaline phosphatase activity or non-specific cross-

reactivities, or related to the need for the bridging antibody to be in excess for adequate binding (Rabbit antibody).

Example 2

OBJECTIVES

The following experiments were done to determine the feasibility of Ki-67 immunostaining of cervical cytology, to determine the sensitivity, specificity and predictive values of Ki-67 immunostaining in predicting cervical dysplasia and to investigate the role of Ki-67 immunostaining in the triage of mildly abnormal cervical cytology.

MATERIALS AND METHODS

Approval for the study was obtained from the institutional R colposcopy because of abnormal cytology Review Board of Thomas Jefferson University Hospital. Patients referred for were offered participation and signed informed consent. Colposcopic referral was for all HGSIL, LGSIL, AGUS cytology. ASCUS smears qualified as favoring neoplasia according to the 1991 Bethesda classification were also referred and underwent Colposcopic examination. ASCUS smears qualified as representing reactive changes are followed by repeat cytology. Smears were obtained from 124 non-pregnant patients at the time of colposcopy. The referral cytology was used as the basis for final comparison to K-1-67 and histology.

Air-dried cervical/endocervical smears were obtained from each patient, and placed onto either Neoprene-coated slides, or, in a few patients, uncoated glass slides. Neoprene (polychloroprene, Aldrich Chemical Company, Milwaukee, Wisconsin) is a chemical that increases cellular adhesion. Exfoliated endocervical material was smeared onto half of the slide from a CYTOBRUSH® sample collection device (MedScand AB, Hollywood FL) and the exocervical sample was smeared onto the other half of the slide from an Ayre spatula.

Air-dried slides were transported to the immunopathology laboratory, desiccated, acetone-fixed, and stored frozen at -70° until stained. MIB-1 antibody to the Ki-67 antigen was obtained from Immunotech (Westbrook, ME). Shandon Cadenza Immunostainers were used to perform the

immunostaining, employing the avidin-biotin peroxidase method and 3, 3'-diaminobenzidine (DAB)/H_2O_2 (Polysciences, Warrington, PA) as the chromogen. A dilute solution of hematoxylin was used as a counterstain.

Slides were evaluated by a cytopathologist who had no knowledge of the results of histologic biopsy or Papanicolaou smears from the patients. The MIB-1 smears were screened by optical microscopy in a manner identical to that of traditional Papanicolaou-stained cervical smears. Slides were evaluated for the presence of nuclear immunoreactivity in squamous cells. In these smears, the reactive nuclei stained dark brown, and were easily discernible at low power from nonreactive, light blue epithelial cell nuclei.

The test was positive when MIB-1 staining was identified in abnormally enlarged squamous nuclei. Nuclear enlargement was defined as a nuclear diameter at least two and half times that of a normal intermediate squamous cell nucleus. Immunoreactivity confined to the nucleolus, endocervical reactivity, or decoration of naked nuclei, devoid of cytoplasm were considered insufficient for positivity. When nuclear decoration was identified in rare cells with minimal nuclear enlargement (1.5 to 2.5 times normal intermediate cell nucleus), the test was considered equivocal. These equivocal results were treated as positive in statistical analysis.

Clinical data collected on each patient including age, parity, smoking history, previous abnormal pap smears or treatment, results of Papanicolaou smear, Colposcopic interpretation, cervical biopsies, and MIB-1 immunostaining, were entered onto a computerized database and analyzed statistically. Univariate and multivariate logistic regression analyses were performed using the SAS statistical package. Odds ratios and 95% confidence intervals were computed as well as sensitivity, specificity, positive and negative predictive values.

RESULTS

Referral cytology was ASCUS, favoring neoplasia (42), AGUS (3), LGSIL (56), and HGSIL (23). The association between

referral cytology and final histologic diagnoses are seen in Table 1.

The average age of these 124 patients was 27.5 (13-62) years. The average parity 1.3 (range 0-6). Thirty-five patients were Caucasian, 84 were African-American and 5 were Hispanic. In this group of patients, 62 (50%) were referred for first abnormal cytology and 62 (50%) had a history of abnormal pap smears or treatment for dysplasia. Forty-three (34.7%) were smokers and 81 (65.3%) were non-smokers. Patients with greater than CIN 2 lesions were treated with loop excision procedures and the final pathology is reported as the worst lesion encountered on either colposcopically directed biopsy or loop excision.

Ki-67 immunoreactivity in relation to final histologic diagnosis is seen in Table 2. All smears were considered satisfactory for evaluation, although some were limited by a scant or absent endocervical component and others were limited by scant cellularity. No smears were considered limited by obscuring inflammatory cells, because these cells were not immunoreactive. Enlarged squamous cell nuclei stained dark brown and were identified without difficulty, even in the presence of numerous light blue neutrophils.

Analysis of the results in the 124 patients who underwent biopsy showed that Ki-67 immunoreactivity had a sensitivity (.89), a specificity (.65), a positive predictive value (.60) and a negative predictive value (.91) in detection of high grade CIN. In this population, the prevalence of high grade CIN was 0.37. In 101 patients with ASCUS, AGUS, and LGSIL, Ki-67 immunostaining demonstrated sensitivity (.96), specificity (.67), positive predictive value (.49) and negative predictive value (.98) in detection of high grade CIN. In this population, the prevalence of high grade CIN was 0.24.

Twenty five patients with ASCUS, AGUS and LGSIL were found to have CIN 2 and CIN 3 on their final histology. Ki-67 immunostaining was positive in 24 of 25 of these patients.

Univariate logistic recession analysis (Table 3) demonstrated that Ki-67 immunostaining was a significant

predictor of high grade cervical pathology (odds ratio 15.5 [95% Cl 5.5 - 43.8]). Additionally, Ki-67 immunostaining was a significant predictor in multivariate analysis (odds ratio 21.5 [95% CI 5.0 - 92.0]) adjusting for age, parity, referral cytology, history of previous treatment, and smoking (Table 4).

DISCUSSION

The appropriate management of patients with mildly abnormal pap smears is not clearly defined. Methods for triaging mildly abnormal smears such as HPV testing, and cervicography are being tested. Colposcopy of all patients with low grade smears is a sensitive but expensive technique for follow-up of these patients. Colposcopy is currently performed for HGSEL. Because 3-20% of patients with pap smears demonstrating LGSIL or atypical cells (ASCUS) will have high grade dysplasia colposcopy is often performed in these patients. Because most patients with minor abnormalities on cervical cytology do not have significant cancer precursors, a highly sensitive test to triage patients to follow-up with cytology or immediate colposcopy would be clinically useful. Immunohistochemical staining is relatively inexpensive, provides a measure of the current proliferative activity of these cells, and appears to be a clinically useful method to identify cervical intraepithelial neoplasia.

Ki-67 nuclear staining is observed in normal mucosa only in parabasal and basal cells, and its expression is seen in higher epithelial levels in SIL. Areas of mature and immature metaplasia, while usually staining in a pattern like that of normal mucosa, may exhibit small numbers of cells in the upper epithelial levels. Ki-67 staining of a few cells in smears from patients with squamous metaplasia is generally regarded as negative, since nuclei in squamous metaplasia are not abnormally enlarged. Abnormal nuclear enlargement in cells with high nuclear to cytoplasmic ration can at times be seen in immature metaplasia. We attribute some of our "false positives" to this phenomenon. The difficulty of distinguishing immature metaplastic cells from high grade SIL

by morphologic means is a problem with Papanicolaou staining that could, in some smears, be resolved by Ki-67 staining.

The evaluation of MIB-1 stained slides is a two-step process, involving both morphologic identification of an enlarged squamous nucleus and MIB- I immunoreactivity using a chromogen to mark those cells. The biology of an ASCUS cell is unknown by morphologic evaluation alone; only approximately 30% of ASCUS diagnoses reveal SIL on biopsy. Ki-67 staining provides data on the biology (i.e. proliferative capacity) of the cells. From our data, an adjunctive test such as Ki-67 staining could potentially reduce the number of Colposcopic procedures in biopsy-negative patients by more than 50%. Ki-67 staining using the MIB-1 antibody correlates more strongly with the presence of HPV in SIL than does biopsy alone.

We demonstrated that it is possible to detect Ki-67 immunoreactivity on cervical cytology specimens. We correlated the results of Ki-67 staining with histopathologic diagnosis and found high sensitivity for this adjunctive test. In addition, we have demonstrated statistically significant correlation between Ki-67 antigen positivity and occurrence of high grade cancer precursors in cervical biopsies.

This test maybe useful in the distinguishing which patients with minor (ASCUS, LGSIL) cytologic abnormalities harbor high grade histologic lesions. We were able to identify 24 of 25 patients with low grade cytology subsequently found to have high grade disease at colposcopy.

HPV/CIN1 lesions were considered as negative for disease in the statistical analysis. While it is recognized that a small percentage of these patients may develop high precursors or cancer, observation of these lesions is an acceptable modality. If no treatment of these patients is planned then detection of such lesions is less important.

False negative testing may reflect the biologic diversity of CIN lesions or simply sampling errors. Ki-67 staining was negative in five patients with histologic changes $\geq$ CIN 2. Four of these five patients ordinarily-would have been

triaged to colposcopy based on HGSIL changes, limiting the false negative results to one patient.

It is noted that the prevalence of CIN is high in this population. Ki-67 staining may demonstrate less predictive value if used in a population with large numbers of inflammatory lesions. However, in previous studies of atypical smears with reactive changes, low rates of high grade precursors were discovered allowing for follow-up with cytology only. Adjunctive testing is probably not necessary in this group of patients with such low incidence of CIN.

The routine Papanicolaou smears were not re-evaluated in this group of patients. AU previous cytology were abnormal and therefore patients underwent colposcopy. The incidence of high grade histology within a group of patients with low grade histology has been previously documented. Standard screening maybe unable to predict histology. Ki-67 staining may identify patients with high grade histology.

This study demonstrates the ability to perform Ki-67 in cervical cytology with a high sensitivity to detect high grade CIN.

TABLE 1

Final Histology and Referral Cytology in 124 Patients

	AGUS (3)	ASCUS (42)	LGSIL (56)	HGSIL (23)
Negative (31)	1 (33.3%)	17 (40.4%)	13 (23.2%)	0(0.0%)
HPV (34)	1 (33.3%)	15 (35.7%)	17 (30%)	1 (4.35%)
CIN 1 (13)		5 (12.0%)	7 (12.5%)	1 (4.35%)
CIN 2 (19)		3 (7.1%)	8 (14.3%)	8 (34.8%)
CIN 3 (25)	1 (33.3%)	2 (4.8%)	11 (20%)	11 (47.8%)
Invasion (2)		0	0	2 (8.7%)

TABLE 2

Comparison of Pathology and Ki-67 Antigen Staining in 124 Patients

	Negative	Equivocal	Positive
Biopsy			
Normal (30)	19	8	3
HPV (35)	13	5	17
CIN 1 (13)	6	0	7
CIN 2 (18)	3	1	14
CIN 3 (26)		1	25
Invasion (2)			2
TOTAL	41	15	68

TABLE 3
Univariate Analysis for Final Pathology ≥ CIN 2

Factor	Odds ratio	95% CI	p value
Ki-67 positivity	15.5	5.5-43.8	0.0001
Smoking	2.1	1.0-4.6	0.05
Parity	1.2	0.4-3.4	0.8
Age (≤ 30 vs. >30)	1.5	0.6 -3.3	0.37
Referral cytology (HGSIL vs. Atypical	0.015	0.003 - 0.08	0.0001
Referral cytology (HGSIL vs. LGSIL)	0.05	0.01-0.23	0.0001
History of previous abnormal pap or treatment	0.9	0.4 - 1.8	0.7

TABLE 4
Multivariable Analysis

Factor	Odds Ratio	95% CI	p value
Ki-67 positivity	21.5	5.0-92	0.0001
Smoking	1.7	0.5 - 5.6	0.39
Parity	1.0	0.2 - 5.1	0.98
Age (≤ 30 vs. > 30)*	3.3	0.7-15.3	0.11
Referral cytology (HGSIL vs. Atypical	0.014	0.002 - 0.1	0.0001
Referral cytology (HGSIL vs. LGSIL)	0.04	0.006-0.25	0.0006
History of previous abnormal pap or treatment	0.8	0.2 - 2.6	0.7

*Because age is correlated with smoking and parity, it was a significant predictor (p=0.02) when entered into a model which excluded smoking and parity.

Example 3

Summary

MIB-1 (Ki-67) stains squamous intraepithelial lesions (SIL) in both histologic and cytologic preparations. The objective of this study was to determine if PAPNET could be utilized to identify MIB-1+ cells in cervical smears.

100 MTB-1 stained smears (ABC method, diaminobenzidine chromogen, hematoxylin counterstain) were submitted for analysis by PAPNET. Routine microscopic evaluation revealed MIB-1+ squamous cells in 57 smears; 49 were biopsied: all but one revealed SIL. Smears from the other 43 patients were devoid of MIB-1 positive squamous cells by routine evaluation; 35 patients had previous abnormal Pap smears - all but six had followup smears or biopsies that were

negative. Of the latter six, three had persistent atypical smears, and three had SIL on biopsy.

PAPNET detected MIB-1+ squamous cells in all 57 smears where they had been identified by manual screening. PAPNET identified MIB-1+ squamous cells in four of 43 smears considered negative by manual screening; three of these four were from the group of six with persistent atypical smears or SIL on biopsy.

Without alteration of its algorithmic or neural network processing; the PAPNET system detected MIB-1+ cells in immunostained cervical smears. This automated detection system, combined with MIB-1 immunostaining for SIL, offers a largely unexplored technology to analyze gynecologic smears.

METHODS

Most of the patients enrolled for this study had previous abnormal Pap smears. At the time of routine gynecologic examination or colposcopic examination, air-dried cervical smears were obtained from patients after signing informed consent. The air-dried smears were either frozen at -70°C or immunostained immediately after acetone fixation. MIB-1 antibody to Ki-67 was used at a dilution of 1:50 (Immunotech, Westbrook, ME). The ABC method was used for immunostaining, which was performed on Shandon Cadenza immunostainers 3,3 diaminobenzidine was the chromogen and hematoxylin was the counterstain.

From a pool of 302 MIB-1 stained cervical smears, 100 were chosen for analysis by PAPNET. All of the slides had been previously screened manually. Smears containing any MIB-1 reactive enlarged squamous nuclei were regarded as positive. Results of the manual screening were noted on the. slide trays, but cytologic or histologic follow up was available only on a separate computer database which was not accessed prior to slide selection and PAPNET analysis.

In this pool of 100 slides, 57 were manually screened as positive, and 43 as negative for MIB-1 expression.

Details of the neural network processing methodology are disclosed in Mango LJ. Neuromedical Systems, Inc. Acta Cytol

1996;40: 53-59;13; Mango LJ. Cancer Lett 1994;77:155-162; and Mango LF, Herriman JM. The PAPNET cytological screening system. In Compendium on the Computerized Cytology and Histology Laboratory. Edited by GL Wied, PH Bartels, DL Rosenthal, U Schenck. Chicago, Tutorials of Cytology, 1994, pp 320-334; which are each incorporated herein by reference). The PAPNET system has been approved by the Food and Drug Administration for clinical use as a rescreening device to supplement conventional screening of Papanicolaou-stained cervical smears, but its use for other purposes is investigational.

In brief, slides were scanned at Neuromedical Systems, Inc., by their computer system which uses both algorithmic and neural network processing. The computer selects 128 cell scenes, identified as potentially abnormal, which are projected onto a high-resolution monitor at a PAPNET review station and interpreted by the operator. Slide coordinates are provided so that manual microscopic review of suspicious areas can be performed.

For the purposes of this study, slide review was considered positive by PAPNET whenever MIB-1 reactive enlarged squamous nuclei were selected by the computer as an abnormal cell scene and projected onto the monitor at the review station. Manual microscopic review was performed on smears at the discretion of the reviewer. The objective of this study was to determine if the computerized technology offered d by PAPNET could be utilized to identify MIB-1 positive cells on cervical smears stained by immunocytochemical methods.

RESULTS

PAPNET analysis identified MIB-1 reactive squamous nuclei in all 57 smears that had been manually screened as positive. In smears that had large numbers of MIB-1 reactive squamous nuclei, many of the cell scenes on the computer monitor displayed MIB-1 reactive cells. Only a few MIB-1 reactive cells were captured on the monitors from smears with few MIB-1 reactive cells Thus, the number of cell scenes showing MIB-1 positive cells was proportional to the number seen manually. In addition, MIB-1 negative koilocytotic cells,

morphologically consistent with human papillomavirus cytopathic effect, were captured by the computerized network and projected onto the monitor.

Biopsies were available from 49 of these 57 patients. All but one revealed a squamous intraepithelial lesion (SIL). Table 5 lists clinical and pathologic data analyzed according to MIB-1 reactivity as detected by PAPNET.

Of the 43 smears manually screened as negative, there were four smears for which PAPNET screening identified rare MIB-1 positive squamous cells - missed by manual screening. In one smear, there was a single cluster of seven positive cells. In two smears, there were two positive cells, and in one smear, there was one positive cell.

Clinically, it was known that 35 of these 43 women had previous abnormal Pap smears. All but six had negative follow up smears or biopsies. Of these latter six, three had persistent atypical smears, and three had SIL on biopsy. Of interest, three of these six patients were from the group of four smears above where MIB-1 positive cells were captured by PAPNET analysis but missed by manual screening. Two had SIL on biopsy and one had an abnormal repeat Pap smear. Results of prior or subsequent smears or biopsies were not known for the fourth patient.

Many of the Pap smears from the patients in this study had been interpreted originally as atypical squamous cells of undetermined significance (ASCUS). MIB-1 negative ASCUS was often captured via the neural network and projected as abnormal cells on the monitor screen. MIB-1 negative ASCUS is interpreted as negative using this immunocytochemical staining method.

DISCUSSION

Without alteration of its algorithmic or neural network processing that was originally developed for the Papanicolaou-stained smear the PAPNET system was able to select MIB-1 reactive cells for analysis and interpretation. In addition to identifying MIB-1 positive cells in all 57 smears where they were found manually, it found rare reactive cells

in four smears where they had been missed manually. Because cervical smears contain at least 300,000 cells, and only a few cells in any smear may be abnormal, the PAPNET system has the capacity to ferret out these rare cells and display them for the operator.

MIB-1 expression is seen in histologic sections of SIL. Low grade SIL derived from HPV 16/18 and 31/33/35/novel types provide higher densities of MIB-1 positive cells than HPV 6/11 lesions. (Other data have found this increased proportion of MIB-1 staining only with HPV 16 associated lesions, Resnick M., et al. Hum. Pathol. 1996 27:234-239.) These data provide support for a hypothesis that MIB-1 reactivity is a marker of the capacity for biologic aggression. We attribute the MIB-1-negative koilocytotic cells that were detected to low densities of MIB-1 reactivity associated with low risk HPV types.

This protocol for MIB-1 staining in cervical smears requires an air-dried acetone-fixed smear. Unmasking of antigens is unnecessary using acetone-fixed smears, since there is no known loss of antigenicity secondary to this type of fixation. Previous attempts to stain SIL using MIB-1 in Pap smears were largely unsuccessful for all but "carcinoma in situ", probably because of antigenic loss due to fixation and failure to unmask the antigens completely with microwave techniques (Boon M.E., et al. Eur J Morphol 1994 32:78-85, and Boon M.E., et al. Mod Pathol 1995 8:786-795.)

MIB-1 labels the nuclear antigen Ki-67, a proliferation marker that stains nuclei in the G1, S, G2 and mitotic phases of the cell cycle. Because its expression is a marker of the biology of the cell, i.e. its proliferative capacity, MIB-1 immunoreactivity provides more useful information than morphology alone.

Care must be taken in the interpretation of MIB-1 reactive nuclei in Pap smears, since rare (up to 4 percent) of normal endocervical cell nuclei react. In endocervicitis and microglandular hyperplasia, up to 18 percent of cells react. In endocervical adenocarcinoma, over 57 percent of cells react.

Thus, a thorough familiarity with endocervical cell morphology is critical for an accurate interpretation of these smears.

TABLE 5
PAP SMEAR RESULTS, MIB-1 IMMUNOREACTIVITY FOLLOWING PAPNET EVALUATION, AND FOLLOWUP

	61 MIB-1 POSITIVE		39 MIB-1 NEGATIVE	
	Pap smears	Followup	Pap smears	Followup
Abnormal		51		3*
ASCUS	26		25	
SIL	28		7	
Normal	4	1	7	31**
Unknown	3	9	0	5

* Two patients had persistent ASCUS on Pap smears and one patient had SIL on biopsy.

**There were seven patients whose followup biopsies revealed low grade SIL but followup Pap smears showed no cellular abnormalities. Since these patients were untreated and, with followup, had no clinically significant lesion, we interpret them as "normal". These low grade SIL may represent either regressing, biologically insignificant lesions, or histologically borderline lesions.

CLAIMS

1. A method of identifying an individual with a squamous intraepithelial lesion comprising the steps of:

a) depositing a sample of exfoliated cervical cells from said individual onto a solid support;

b) fixing cells of said sample by non-immunologically neutralizing desiccation or a non-non-immunologically neutralizing fixative,

c) contacting said sample with detectable antibodies that bind to antigens that are expressed by proliferating epithelial cells for a time sufficient and under conditions suitable for said antibodies to form complexes with said antigens present in said sample,

d) removing unbound antibodies from said sample, and

e) inspecting said sample to detect the presence of antibodies bound to antigen in epithelial cells in said sample; wherein the presence of antibodies bound to antigen in epithelial cells in said sample indicates a squamous intraepithelial lesion.

2. The method of claim 1 wherein said solid support is a glass slide.

3. The method of claim 2 wherein said glass slide is selected from the group consisting of: a polychloroprene coated slide, a silanated slide, a poly-L-lysine slide and a positively charged slide.

4. The method of claim 3 wherein said glass slide is a polychloroprene coated slide.

5. The method of claim 2 wherein said glass slide is uncoated.

6. The method of claim 1 wherein said cells of said sample is fixed by air drying.

7. The method of claim 1 wherein said cells of said sample is fixed with a non-immunologically neutralizing desiccant or a non-immunologically neutralizing fixative.

8. The method of claim 1 wherein said sample is contacted with detectable antibodies that bind to an antigen selected from the group consisting of Ki-67, cyclins, p53 and other markers of cellular proliferation.

9. The method of claim 1 wherein said sample is contacted with antibody MIB-1.

10. The method of claim 1 wherein said antibodies are detected by an avidin-biotin peroxidase method, an immunoalkaline phosphatase anti-alkaline phosphatase, streptavadin peroxidase, streptavadin alkaline phosphate, glucose oxidase, fluorescein conjugates or immunogold.

11. A method of identifying an individual with a squamous intraepithelial lesion comprising the steps of:

a) depositing a first sample of exfoliated cervical cells from said individual onto a solid support;

b) depositing a second sample of exfoliated cervical cells from said individual onto a solid support;

c) fixing cells of said first sample with a denaturing fixative that prepares cells for Papanicolaou staining,

d) fixing cells of said second sample by non-immunologically neutralizing desiccation or a non-immunologically neutralizing fixative,

e) contacting said first sample with Papanicolaou stain for sufficient time for cells in said first sample to incorporate stain,

f) washing said first samples to remove unincorporated stain,

g) evaluating staining pattern of cells of said first sample to identify samples as containing normal cells only or a squamous intraepithelial lesion or atypical squamous cells of

undetermined significance, wherein said second sample is evaluated by immunocytochemical assay when said first sample is identified as atypical squamous cells of undetermined significance; said immunocytochemical assay comprises the further steps of

h) contacting said sample with detectable antibodies that bind to antigens that are expressed by proliferating epithelial cells for a time sufficient and under conditions suitable for said antibodies to form complexes with said antigens present in said second sample,

i) removing unbound antibodies from said second sample, and,

j) inspecting said second sample to detect the presence of antibodies bound to antigen in epithelial cells in said second sample; wherein the presence of antibodies bound to antigen in epithelial cells in said second sample indicates a squamous intraepithelial lesion.

12 The method of claim 11 wherein said solid support is a glass slide.

13. The method of claim 11 wherein said cells of said second sample is fixed by air drying.

14. The method of claim 11 wherein said cells of said second sample is fixed with a non-immunologically neutralizing desiccant or a non-immunologically neutralizing fixative.

15. The method of claim 11 wherein said second sample is contacted with detectable antibodies that bind to antigens Ki-67.

16. The method of claim 11 wherein said second sample is contacted with antibodies MIB-1.

17. The method of claim 11 wherein said antibodies are detected by an avidin-biotin peroxidas, an immunoalkaline

phosphatase anti-alkaline phosphatase, glucose oxidase, fluorescein conjugates or immunogold.

18. A method of identifying whether an individual with atypical squamous cells of undetermined significance has a squamous intraepithelial lesion comprising the steps of:

a) identifying said individual with atypical squamous cells of undetermined significance by Papanicolaou smear analysis;

b) depositing a sample of exfoliated cervical cells from said individual onto a solid support;

c) fixing cells of said second sample by non-immunologically neutralizing desiccation or a non-immunologically neutralizing fixative,

d) contacting said sample with detectable antibodies that bind to antigens that are expressed by proliferating epithelial cells for a time sufficient and under conditions suitable for said antibodies to form complexes with said antigens present in said second sample,

e) removing unbound antibodies from said second sample, and,

f) inspecting said second sample to detect the presence of antibodies bound to antigen in epithelial cells in said second sample; wherein the presence of antibodies bound to antigen in epithelial cells in said second sample indicates a squamous intraepithelial lesion.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/17399

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/574, 33/53
US CL :435/7.23, 7.9, 7.92; 436/518, 63, 64, 813
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.23, 7.9, 7.92; 436/518, 63, 64, 813

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MORHENN et al. A Monoclonal Antibody against Basal cells of Human Epidermis, Potential Use in the Diagnosis of Cervical Neoplasia. Jornal of Clinical Investigation. November 1985, Vol. 76, pages 1978-1983, especially the Abstract and the right-hand column of page 1982.	1-18
Y	KOPROWSKA et al. Common Antigenic Sites on Exfoliated Cells derived from Cervical Carcinoma and in Tumor Cells of Nonuterine Origin as Demonstrated by Monoclonal Antibodies in Immunoperoxidase Assay. CANCER RESEARCH, November 1985, Vol. 45, pages 5964-5968, especially page 5967.	1-18

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 23 DECEMBER 1996	Date of mailing of the international search report 15 JAN 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer TONI R. SCHEINER Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/17399

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,782,015 A (ALLISON et al.) 01 November 1988, see the Abstract and column 11, lines 5-44.	1-18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/17399

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, DIALOG (file biochem)
search terms: immunohistochem?, immunocytochem?, immunohist?, cytomorphol?, squamous intraepithelial lesion, SIL, squamous atypia, atypical squamous, ASCUS, carcinoma, neoplas?, cancer, antibod?, prolifer?, cervical, cervix